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CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

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Fort Detrick, Maryland 21702-5012

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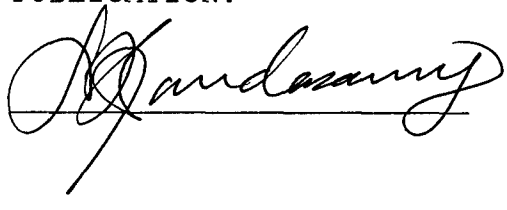

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13. ABSTRACT ***(Maximum 200 Words)***

Breast cancer progression from a hormone-dependent, anti-estrogen sensitive to a hormone-independent, anti-estrogen insensitive phenotype involves the loss of estrogen receptor (ER) expression and the up-regulation of a number of growth factor receptors and/or their ligands, including the epidermal growth factor receptor (EGFR). EGFR is overexpressed in breast cancer and is inversely correlated with ER status in a majority of breast tumors. EGFR overexpression is associated with a more aggressive phenotype and predicts for poor response to endocrine therapy, suggesting up-regulation of EGFR is involved in the progression to a more aggressive, hormone-independent phenotype. Results indicate that a 96bp fragment within the EGFR gene first intron repressed EGFR gene expression in ER+ breast cancer cells. Furthermore, the 96bp intron element demonstrated differential factor binding in ER+ vs. ER- breast cancer cells. Results point to the 96bp intron element as being the optimal repressor element. Disruption of this element and/or the factors with which it interacts results in the abolishment of transcriptional repression. Moreover, it appears that the 96bp element interacts with a cell-specific factor, as well as a factor that is estrogen-regulated. Tanscriptional repression plays a major role in the regulation of EGFR gene expression in hormone-dependent breast cancer cells.

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Table of Contents

THE ROLE OF A FIRST INTRON NEGATIVE REGULATORY ELEMENT IN THE REPRESSION OF EGFR EXPRESSION IN HORMONE-DEPENDENT BREAST CANCER

INTRODUCTION

The progression of breast cancer from an estrogen receptor positive (ER+), hormone-dependent to an estrogen receptor negative (ER-), hormone-independent phenotype involves the loss of ER expression and the up-regulation of a number of growth factor receptors and/or their ligands (1-3). The EGFR is one particular receptor that is overexpressed in breast cancer. There is a noted inverse correlation between the overexpression of ER and EGFR in a majority of breast tumors, being either ER+/EGFR- or ER-/EGFR+ (3-6). However, a significant number of tumors co-express both receptors, being ER+/EGFR+, while the absence of both receptors (ER-/EGFR-) is a rare occurrence (5,6). Individual cells within the population of ER+/EGFR+ tumors overexpress one of the receptors, ER or EGFR, but not both, emphasizing the inverse relationship of ER vs. EGFR in breast cancer (5,6). The overexpression of EGFR, independent of ER status, is indicative of a more aggressive phenotype and predicts for poor response to endocrine therapy (7) and poor survival rate (8), suggesting that up-regulation of EGFR is involved in the progression of breast cancer to a more aggressive, hormone-independent phenotype. Such overexpression of EGFR in breast cancer cells is primarily due to transcriptional control. Both positive and negative regulatory factors appear to play an important role in the regulation of EGFR gene expression in other cell lines and are suspected to do so in breast cancer cell lines (9, 10). The main focus of our laboratory is the investigation of the differential regulation of the EGFR gene in ER+ vs. ER- breast cancer cell lines. Both *in vitro* and *in vivo* experiments performed in our lab have indicated the involvement of regions within the EGFR gene first intron in the enhancement and repression of EGFR gene expression. The overall goal of this project is the identification of negative regulatory elements within the EGFR gene first intron and characterization of mechanisms by which EGFR gene expression is repressed in estrogen-dependent breast cancer.

BODY

Research accomplishments

(see appendices for figures)

The main focus of our laboratory is the investigation of the differential regulation of the EGFR gene in ER+ vs. ER- breast cancer cells. Experiments performed in the lab have indicated regions within the EGFR gene first intron are involved in both the enhancement and repression of EGFR gene expression. To investigate the role of intron 1 regions in the repression of the EGFR gene in ER+ vs. ER- breast cancer cells and to identify the minimal cis-element(s) demonstrating repressive activity, transient transfections were performed utilizing heterologous and homologous promoters. The homologous promoter constructs, designated pJFEC (or pJ), address the ability of intron fragments to regulate transcriptional activity of the EGFR promoter, while the

heterolgous promoter constructs, designated promCAT (or pC), investigate their ability to control transcriptional activity of the SV40 promoter.

Intially, results from transient transfections with reporter constructs had identified a 730 bp region (Figure 1) within the 5' region of the EGFR gene first intron that repressed SV40 transcriptional activity in the ER+/low EGFR expressing MCF-7 breast cancer cells, but not the ER-/high EGFR expressing BT549 breast cancer cells. Moreover, a 305 bp fragment (Figure 1) within this intron region retained repressive activity in MCF-7, but not BT549, breast cancer cells with both the SV40 and EGFR promoters. While other intron regions were observed to be involved in the transcriptional regulation of EGFR gene expression, we focused on identifying the minimal negative regulatory element within the EGFR gene first intron that was required for transcriptional repression in ER+/low EGFR expressing breast cancer cells. Therefore, we concentrated on the 305 bp intron element.

In vitro DNase I footprinting experiments were performed with the 305 bp intron negative regulatory element in order to identify regions of DNA-protein interaction, as well as define DNA sequences involved in these interactions. An altered digestion pattern was visualized upon incubation of the 305 bp intron fragment with MCF-7 nuclear extracts, as compared to probe alone (Figure 2). Numerous regions were protected from digestion with DNase I, designated by rectangles and roman numerals; furthermore, sites with increased sensitivity to DNase I digestion appeared at the boundaries of these footprinted regions, indicated by arrows. These protected regions and hyoersensitive sites are indicative of factor binding. However, the binding of factors *in vitro* may not always reflect the true *in vivo* situation; chromatin conformation and factor binding site accessibilty may act to regulate *in vivo* DNA-protein interactions.

Based on results obtained from in vitro DNase I footprinting experiments, we generated constructs containing smaller intron regions corresponding to the 305 bp intron negative regulatory elements. Utilizing the 56 bp, 96 bp, and 150 bp pJFEC constructs (Figure 1), we localized the major negative regulatory element within the 96 bp intron fragment. Results from transient transfections in breast cancer cells demonstrated that the 96 bp fragment dramatically reduced EGFR transcriptional activity in ER+/low EGFR expressing MCF-7 breast cancer cells, repressing transcription 93% +/- 3.7 ($p<0.001$), but did not repress transcriptional activity in ER-/high EGFR expressing BT549 breast cancer cells (Figure 3 A&B). While the other intron fragments also demonstrated repressive activity in MCF-7 cells, repression was not as dramatic. Moreover, gel shift assays with this fragment and nuclear extracts from these cell lines demonstrated differential shift patterns. The 96 bp fragment interacted with factors within MCF-7 cells producing a shifted complex, migrating slower than that probe alone, that was almost completely absent in the BT549 nuclear extracts (Figure 3C). Nuclear extracts were made from MCF-7 cells that were treated with the anti-estrogen, ICI 182,780 (ICI), 5 days prior to nuclear extract preparation (MCF-7/ICI). It has been demonstrated that treatment of MCF-7 cells with ICI results in increased EGFR levels (11). Gel shift assays performed with MCF-7/ICI nuclear extracts also demonstrated reduced complex formation with the 96 bp intron negative regulatory element (Figure 3C), suggesting that repressor binding and/or activity is estrogen-regulated.

To further delineate the minimal negative regulatory element, a series of constructs were made utilizing multiple oilgonucleotides representing the 96 bp intron negative regulatory element (Figure 1). Averaged results from at least four transient transfection assays with EGFR promoter constructs containing the 59 bp intron element demonstrated that the 59 bp fragment repressed activity 50% +/- 11 ($p<0.001$) in MCF-7 cells (n=4) (Figure 4A) and 62% +/- 11 ($p<0.001$) in BT549 cells (n=6) (Figure 4B). Results from three transient transfections with EGFR promoter constructs containing the 37 bp fragment demonstrated no significant effect in MCF-7 or BT549 cells (Figure 4 A&B). These results suggested that while 59 bp intron fragment repressed EGFR transcriptional activity, it lost its differential repressive activity, repressing EGFR transcriptional activity in both the ER+/low EGFR expressing MCF-7 cells and the ER-/high EGFR expressing BT549 cells.

The EGFR promter-intron constructs containing the intron regions FP6 and FP7 correspond to specific footprinted regions, VI and VII respectively (Figure 2), within the 305 bp intron element. In MCF-7 breast cancer cells, results from three transient transfections of these constructs containing the oligonucleotides demonstrated that F1, F3, FP6, and FP7 had no significant effect on EGFR transcriptional activity (Figure 4A). In BT549 cells, results from three transient transfections demonstrated that F1 and F3 had no significant effect on transcriptional activity, while FP6 and FP7 decreased EGFR transcriptional activity 28% +/- 0.2 ($p=0.04$) and 66.7% +/- 0.2 ($p=0.002$), respectively (Figure 4B).

The 59 bp and 37 bp fragments were used as probes in gel shift assays in order to investigate factor binding to these two elements, comprising the 96 bp intron fragment. Incubation of the 59 bp fragment with MCF-7 nuclear extract resulted in a single shifted complex, relative to probe alone, which was somewhat reduced in BT549 nuclear extracts and appeared to be unchanged in MCF-7/ICI nuclear extracts (Figure 5A). Incubation of the 37 bp fragment with MCF-7 nuclear extract resulted in two shifted complexes (complexes I & II), relative to probe alone. While complex I was similar in all nuclear extracts, complex II was greatly reduced in BT549 nuclear extracts and MCF-7/ICI nuclear extracts (Figure 5B). While the 37 bp fragment had no significant effect on EGFR transcriptional activity in MCF-7 or BT549 breast cancer cells, it demonstrated differential DNA-protein complexes (complex II). Our results suggested that the 37 bp intron region may recruit a cell-specific factor that modulates the activity of the factor(s) interacting with the 59 bp intron element.

Gel shift assays utilizing F1 and F3 as probes displayed similar binding patterns between ER+ vs. ER- nuclear extracts. However, gel shifts with probes corresponding to FP6 and FP7 demonstrated differences in factor binding. Incubation of nuclear extracts with the FP6 or FP7 probe demonstrated an estrogen-dependence of binding (Figure 5 C&D); DNA-protein complexes formed between the probes and MCF-7 nuclear extract are dramatically decreased in BT549 nuclear extracts. Similarly, complex formation was reduced in nuclear extracts made from ICI treated MCF-7 cells (5 C&D). FP6 and FP7, sub-fragments of the 96 bp intron negative regulatory element, retained the estrogen-dependence of binding, like the 96 bp fragment. Division of the 96 bp intron negative regulatory element into smaller fragments appeared to result in the loss of repressive

activity in the MCF-7 breast cancer cells. Our results suggest that the 96 bp intron element is the optimal repressive element, and that a complex consisting of regulatory factors interacts with this element. Disruption of this element results in protein complex disruption, and therefore, loss of transcriptional regulation.

Our results establish a difference in transcriptional activity and DNA-protein interactions with the intron negative regulatory element between ER+ vs. ER- breast cancer cells. Moreover, contrasting results were obtained in electrophoretic mobility shift assays with nuclear extracts isolated from ICI treated MCF-7 cells, as compared to native MCF-7 cells. Therefore, the role of estrogen-regulation of this intronic repressor element was investigated. Transient transfection assays with the EGFR promoter-CAT construct containing the 305 bp intron negative regulatory element were performed in the absence and presence of the pure anti-estrogen, ICI 182,780 (ICI). As previously demonstrated by Yarden *et. al.* (11), treatment with ICI results in increased EGFR levels, while the continual presence of estrogen repressed EGFR levels. Treatment with 10^{-7}M ICI for 48 hours post-transfection resulted in the loss of transcriptional repression originally observed with the 305 bp intron negative regulatory element. ICI treatment increases the transcriptional activity of the 305 bp intron element 7.9-fold over the EGFR promoter alone in the presence of ICI. Moreover, ICI treatment results in the overall increase of the 305 bp transcriptional activity, producing a 57-fold increase of activity over that of the 305 bp element in untreated MCF-7 cells (Figure 6). These results strongly suggest that transcriptional repression mediated through the 305 bp intron negative regulatory element is estrogen-dependent.

Southwestern analysis (or DNA-protein blotting) was performed to investigate proteins interacting with the 305 bp negative regulatory element. We analyzed nuclear extracts from a representative panel of ER+ vs. ER- breast cancer cell lines in order to examine the estrogen-dependence of the repressor with respect to cell-specificity. The 305 bp intron negative regulatory element interacted with two proteins, around 130 kDa and 35 kDa, present in nuclear extracts from both ER+ and ER- breast cancer cells, although at varying levels (Figure 7). Whereas *in vitro* DNase I footprinting identified numerous factors interacting with the 305 bp intron fragment, Southwestern analysis identified a subset of these factors. The inherent limitations of Southwestern analysis limit the detection of interacting factors; proteins that require protein-protein interactions (protein complexes) or that need to be in their native conformation are not detected. Consequently, while results from Southwestern analysis are inconclusive, they do suggest that transcriptional regulation mediated through the 305 bp intron negative regulatory element is complex and involves multiple factors.

Transcriptional regulation of EGFR gene expression is complex and results from the balance between both positive and negative transcription factors. In breast cancer, EGFR transcriptional activity is regulated through factor binding sites in the EGFR gene promoter, as well as through intron 1 elements. We have identified a 305 bp negative regulatory element within the EGFR gene first intron which demonstrates repressive activity with both heterologous and homologous promoter/CAT constructs in the ER+/low EGFR expressing MCF-7 breast cancer cells, but not in the ER-/high EGFR expressing BT549 breast cancer cells. Furthermore, a 96 bp fragment within this element demonstrates differential binding to factors in MCF-7 vs. BT549 nuclear extracts. This

binding activity correlates with transcriptional activity; the 96 bp intron fragment represses EGFR transcriptional activity in MCF-7 cells but not in BT549 cells. Results from transient transfections and electrophoretic mobility shift assays, as well as speculative data from Southwestern analysis, suggest that multiple factors are involved in the transcriptional repression mediated by the 96 bp intron element. The underlying mechanism by which repression is achieved may involve DNA binding site competition and/or recruitment of auxiliary factors, including a cell-specific regulator. Moreover, one or more of the factors interacting with the 96 bp intron element and/or the associated transcriptional activity appears to be estrogen-regulated, tightly linking the progression of breast cancer to a hormone-independent phenotype and the loss of transcriptional repression during this process.

This project provided Dr. Wilson with training in the areas of gene regulation and breast cancer. I have completed my doctoral thesis and am subsequently working as a post-doctoral fellow with Dr. Trevor Archer at the National Institute of Environmental Health Sciences in Research Triangle Park, North Carolina. I am continuing my pursuit of breast cancer research concentrating on steroid hormone receptors and regulation of gene expression. I have finished writing a manuscript detailing the work and results of my thesis project and plan to submit it for publication.

KEY RESEARCH ACCOMPLISHMENTS

A 305 bp region within the EGFR gene first intron demonstrates repressive activity with both heterologous and homologous promoter/CAT constructs in the ER+/low EGFR expressing MCF-7 breast cancer cells, but not in the ER-/high EGFR expressing BT549 breast cancer cells.

Multiple factors within MCF-7 nuclear extracts interact with the 305 bp intron negative regulatory element, as determined by in vitro DNase I footprint experiments. These factors may be involved in the transcriptional activity displayed by this intron element.

A 96 bp fragment within the 305 bp intron region contains the major negative regulatory element responsible for repressive transcriptional activity. Furthermore, this element appears to interact with a complex of regulatory factors, and upon disruption of the element and/or factor complex, transcriptional repression is abolished.

The factors involved in DNA-protein interactions with the 96 bp intron negative regulatory element appear to be comprised of a cell-specific factor, as well as a factor that is estrogen-regulated. Transcriptional repression is abrogated when ER+ cells are treated with the anti-estrogen ICI.

The transcriptional regulation of EGFR gene expression in ER+ breast cancer cells is complex and a balance between positive and negative regualtory factors. The mechanism of transcriptional repression exerted by the 96 bp intron repressor element may involve competition for factor binding sites and/or recruitment or transcritional co-factors (auxiliary factors) involved in mediating transcriptional repression.

REPORTABLE OUTCOMES

manuscript in preparation: Wilson, M.A and Chrysogelos, S.A. "Identification and characterization of a negative regulatory element within epidermal growth factor receptor gene first intron in hormone-dependent breast cancer cells."

Wilson, M.A and Chrysogelos, S.A. Negative regulation of EGFR transcriptional activity in hormone-dependent breast cancer cells. DoD USAMRMC Era of Hope Meeting, Atlanta, Georgia, June 8-11, 2000.

Dissertation submitted to the Faculty of the Graduate School of Arts and Sciences of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry and Molecular Biology.
"Transcriptional Repression of Epidermal Growth Factor Receptor Gene Expression in Hormone-Dependent Breast Cancer Cells." November, 1999.

Post-doctoral fellow at the National Institutes of Environmental Health Sciences (NIEHS) under Dr. Trevor Archer.

MANUSCRIPTS AND ABSTRACTS ATTACHED

Abstract: Wilson, M.A and Chrysogelos, S.A. Negative regulation of EGFR transcriptional activity in hormone-dependent breast cancer cells. DoD USAMRMC Era of Hope Meeting, Atlanta, Georgia, June 8-11, 2000.

CONCLUSIONS

EGFR exhibits an inverse correlation with ER expression in the majority of breast cancers, predicting for poor response to endocrine therapy and poor survival rate. Inappropriate overexpression of EGFR in breast cancer is associated with a more aggressive cancer, suggesting that EGFR up-regulation is involved in the progression of breast cancer to a more aggressive, hormone-independent phenotype. Primarily, identification of transcription factors regulating EGFR gene expression has been performed in HeLa and A431 cells. Moreover, the majority of transcription factor binding sites investigated reside in the EGFR gene promoter. Experiments performed in our laboratory, both *in vivo* and *in vitro*, identified regions within the EGFR gene first intron which appear to be involved in the differential regulation of EGFR gene expression in ER+ vs. ER- breast cancer cell lines. In this study, we identify and characterize a negative regulatory element within the EGFR gene first intron demonstrating differential transcriptional activity and DNA-protein interactions in ER+ vs. ER- breast cancer cell lines.

Transcriptional regulation of EGFR gene expression is complex and results from the balance between both positive and negative transcription factors. In breast cancer, EGFR transcriptional activity is regulated through factor binding sites in the EGFR gene promoter, as well as through intron 1 elements. We have identified a 305 bp negative regulatory element within the EGFR gene first intron which demonstrates repressive activity with both heterologous and homologous promoter/CAT constructs in the ER+/low EGFR expressing MCF-7 breast cancer cells, but not in the ER-/high EGFR expressing BT549 breast cancer cells. Furthermore, a 96 bp fragment within this element demonstrates differential binding to factors in MCF-7 vs. BT549 nuclear extracts. This binding activity correlates with transcriptional activity; the 96 bp intron fragment represses EGFR transcriptional activity in MCF-7 cells but not in BT549 cells. Results from transient transfections and electrophoretic mobility shift assays, as well as speculative data from Southwestern analysis, suggest that multiple factors are involved in the transcriptional repression mediated by the 96 bp intron element. The underlying mechanism by which repression is achieved may involve DNA binding site competition and/or recruitment of auxiliary factors, including a cell-specific regulator. Moreover, one or more of the factors interacting with the 96 bp intron element and/or the associated transcriptional activity appears to be estrogen-regulated, tightly linking the progression of breast cancer to a hormone-independent phenotype and the loss of transcriptional repression during this process.

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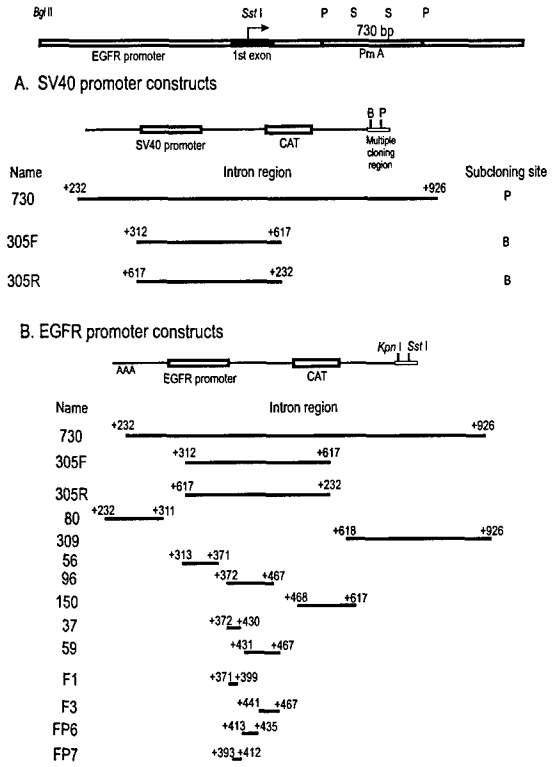
Figure 1.



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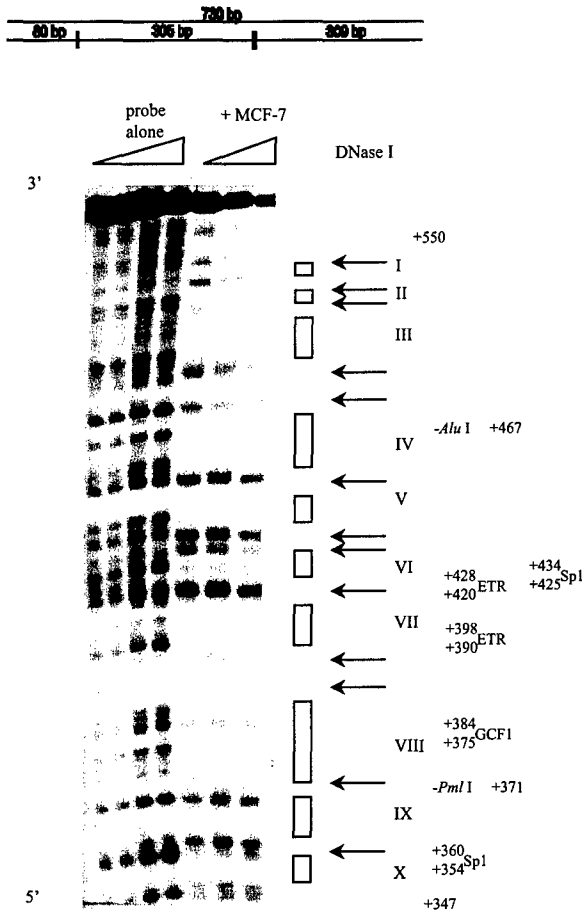


Figure 7.

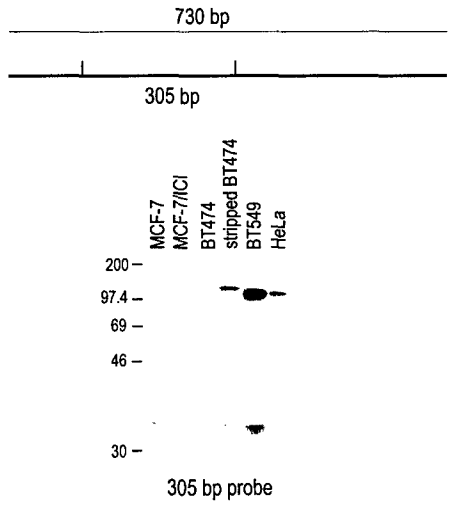


Figure 1. *Depiction of EGFR gene first intron fragments and promoter/CAT constructs.* Map of ~2.5 Kb of the EGFR gene including the proximal promoter, first exon, and ~1.5 Kb of the firs intron is shown. Schematic representation of EGFR gene first intron elements subcloned into reporter constructs used intransient transfections to investigate transcriptional activity. The positions of the EGFR intron fragments indicated are relative to the translational start site. *A.)* SV40 promoter-EGFR intron constructs. EGFR inron elements were subcloned into the parental SV40-promoter-CAT constuct, designated promCAT (or pC) as described in materials and methods. Sites of subcloning are indicated. *B.)* EGFR promter-intron constructs. EGFR intron elements were subcloned into the parental EGFR promoter-CAT construct, designated pJFEC (or pJ) as described in material and methods. Intron elements were subcloned into the *Kpn* I and *Sst* I restriction sites. F and R refer to the forward and reverse orientation, respectively, of subcloned intron fragments. A, *Alu* I; B, *BamH* I; P, *Pst* I; Pm, *Pml* I; and S, *Sau3A* I.

Figure 2. *Multiple factor binding sites localize within the 305 bp intron negative regulatory element.*
A schematic of the 305 bp intron negative regulatory depicting the footprint probe used to define specific regions of DNA-protein interaction. The 305 bp probe, Sau3A I to Sau3A I intron fragment, was incubated with increasing concetrations of DNase I (0 to 25 ng) in the absence (probe alone) of presence of 40 ug MCF-7 nuclear extract (+ MCF-7). Ten distinct regions of protection were identified within the 305 bp intron negative regulatory element, designated by rectangles and roman numerals. Arrows designate regions of increased DNase I sensitivity. Putative binding sites, as well as postions within the 305 bp intron element, are indicated.

Figure 7. *The 305 bp intron negative regulatory element interacts with multiple factors in ER+ vs. ER- nuclear extracts.*
Map of the 305 bp negative regulatory element used as the probe to identify protein-DNA interaction. Southwestern analysis (DNA-protein blotting) was performed to examine factors interacting with the 305 bp fragment which demonstrates repressive transcriptional activity. Nuclear extracts from a panel of breast cancer cell lines were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with the ^{32}P-labeled 305 bp DNA fragment. BT474 cells are ER+ and express considerably higher levels of EGFR than MCF-7 cells. The role of estrogen was investigated by treating MCF-7 cells with an anti-estrogen, ICI, or by estrogen depleting BT474 cells (stripped BT474). MCF-7/ADR cells are ER-/high EGFR expressors. HeLa cells are human cervical carcinoma cells.

Figure 3.

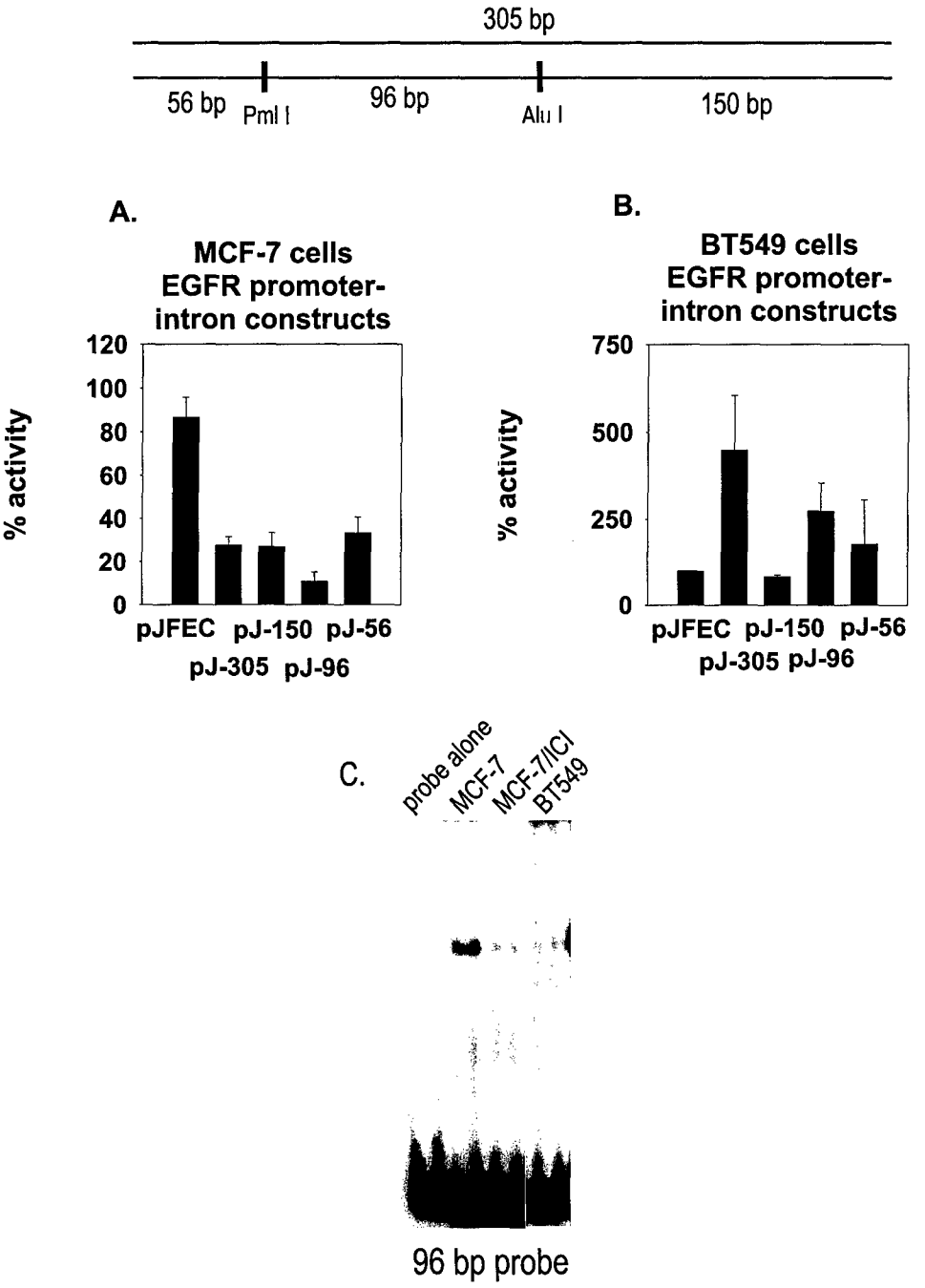

305 bp
56 bp
Pml I
96 bp
Alu I
150 bp
A.
MCF-7 cells
EGFR promoter-intron constructs
% activity
120
100
80
60
40
20
0
pJFEC
pJ-150
pJ-56
pJ-305
pJ-96
B.
BT549 cells
EGFR promoter-intron constructs
% activity
750
500
250
0
pJFEC
pJ-150
pJ-56
pJ-305
pJ-96
C.
probe alone
MCF-7
MCF-7/ICI
BT549
96 bp probe

Figure 3. *Localization of the minimal intron negative regulatory element.*
Schematic of the 305 bp intron negative regulatory element and its subfragments. *A.)* Transcriptional activity of EGFR promoter-intron constructs containing the smaller subfragments in ER+/low EGFR expressing MCF-7 breast cancer cells. *B.)* Same as in A.), except in ER-/high EGFR expressing BT549 breast cancer cells. p values: *, p=0.01; **, p=0.002; ***, p=0.003; and ****, p=0.004. C.) Gels shift assay with the 96 bp intron fragment. 5ug of nuclear extracts were incubated with a probe corresponding to the 96 bp minimal intron negative regulatory element. Shifted DNA-protein complexes demonstrate a slower migration compared to the probe alone.

Figure 4.

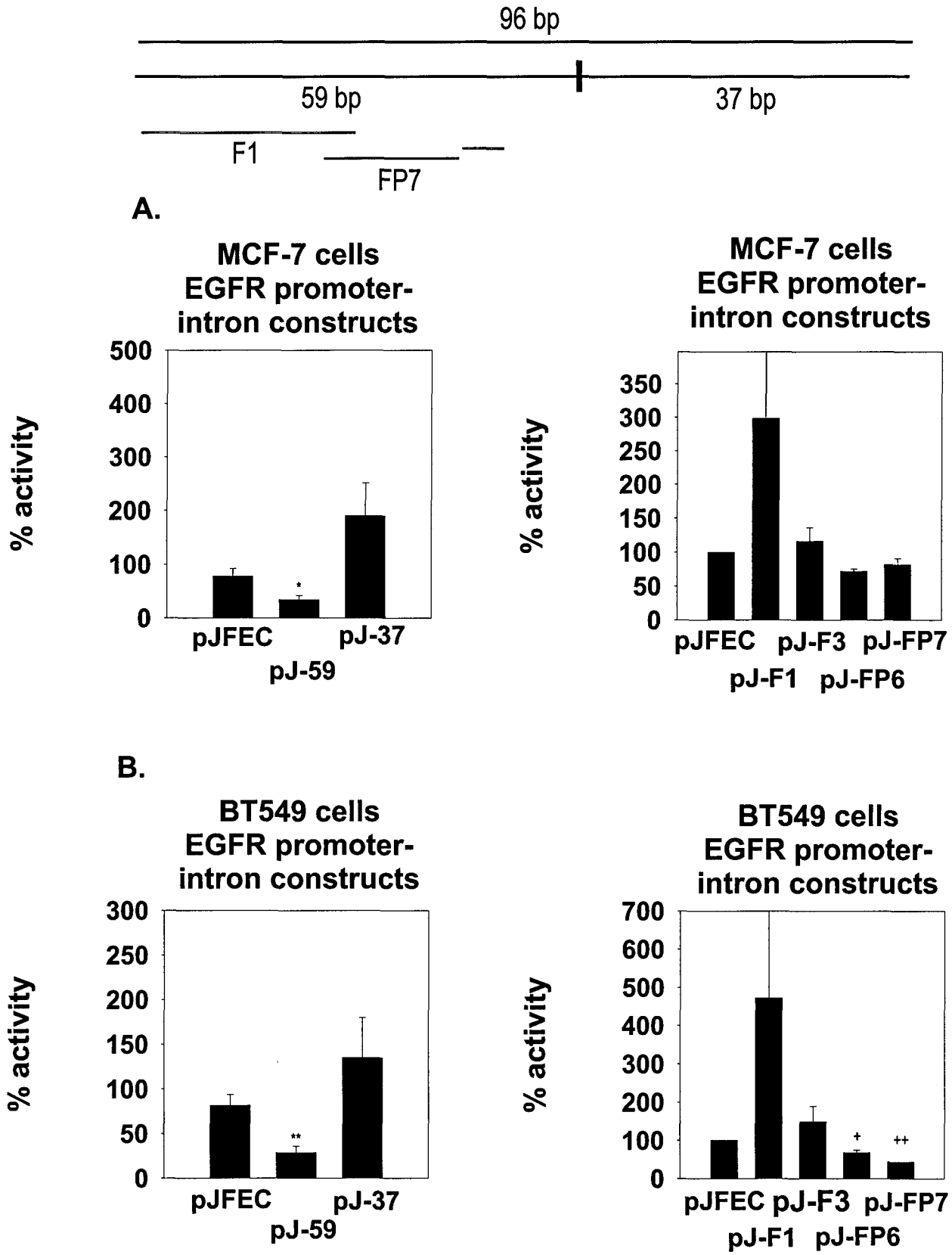

96 bp
59 bp
37 bp
F1
FP7
A.
MCF-7 cells
EGFR promoter-
intron constructs
% activity
500
400
300
200
100
0
pJFEC
pJ-37
pJ-59
MCF-7 cells
EGFR promoter-
intron constructs
% activity
350
300
250
200
150
100
50
0
pJFEC
pJ-F3
pJ-FP7
pJ-F1
pJ-FP6
B.
BT549 cells
EGFR promoter-
intron constructs
% activity
300
250
200
150
100
50
0
pJFEC
pJ-37
pJ-59
BT549 cells
EGFR promoter-
intron constructs
% activity
700
600
500
400
300
200
100
0
pJFEC
pJ-F3
pJ-FP7
pJ-F1
pJ-FP6

Figure 4. *Disruption of the minimal intron negative regulatory element results in loss of differential transcriptional activity.*
Map of oligonucleotides representing the 96 bp intron negative regulatory element. Transcriptional activity of EGFR promoter-intron constructs containing these intron regions in *A.)* ER+/low EGFR expressing MCF-7 brease cancer cells and *B.)* ER-/high EGFR expressing BT549 breast cancer cells. p values: *, $p<0.09$; **, $p=0.02$; +, $p=0.04$; and ++, $p=0.002$.

Figure 5.

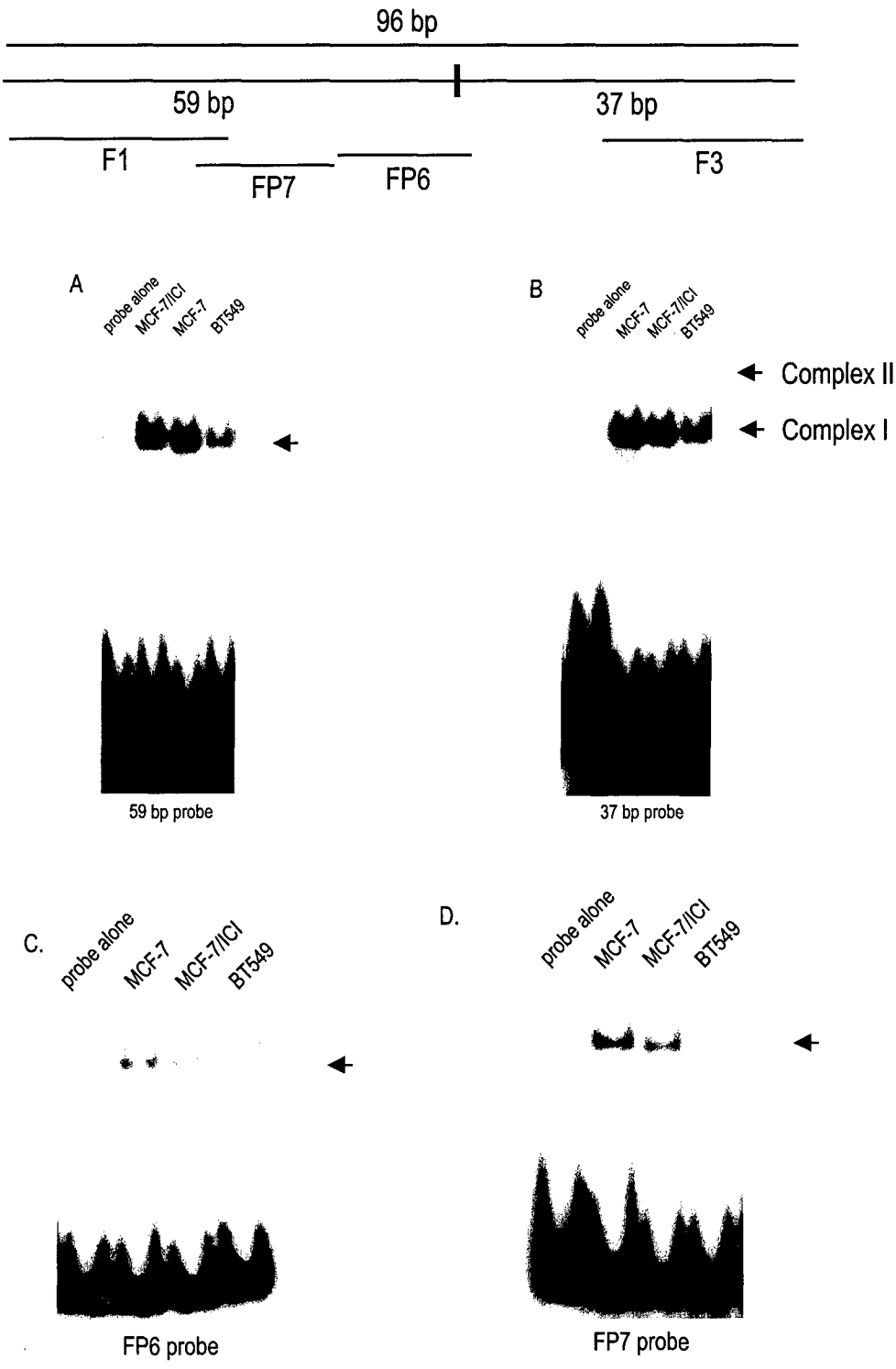
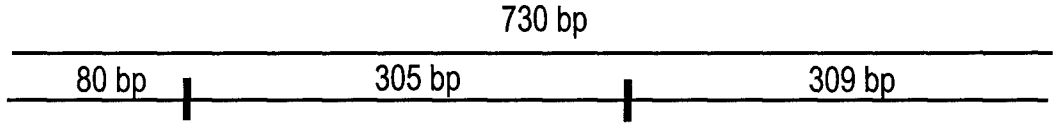

96 bp
59 bp
37 bp
F1
FP7
FP6
F3
A
probe alone
MCF-7/ICI
MCF-7
BT549
B
probe alone
MCF-7
MCF-7/ICI
BT549
Complex II
Complex I
59 bp probe
37 bp probe
C.
probe alone
MCF-7
MCF-7/ICI
BT549
D.
probe alone
MCF-7
MCF-7/ICI
BT549
FP6 probe
FP7 probe

Figure 5. *EGFR intron elements demonstrate differentail DNA-protein complex formation with nuclear extracts from ER+ vs. ER- nuclear extracts.*
Map of oilgonucleotides spanning the 96 bp intron negative regulatory element used as probes in gel shift assays. In all cases, 5ug of nuclear extracts were incubated with probes corresponding to regions within the EGFR gene first intron, including: *A.)* the 59 bp element; *B.)* the 37 bp element; *C.)* FP6, corresponding to footprint region VI (Figure 4); and *D.)* FP7, corresponding to footprint region VII (Figure 4). DNA-protein complexes were analyzed on 6% non-denaturing polyacrylamide gels. Nuclear extracts were made from MCF-7 cells treated with ICI 182,780 for 5 days prior to harvest (MCF-7/ICI). Arrows designate protein-DNA complexes.

Figure 6.


730 bp
80 bp
305 bp
309 bp

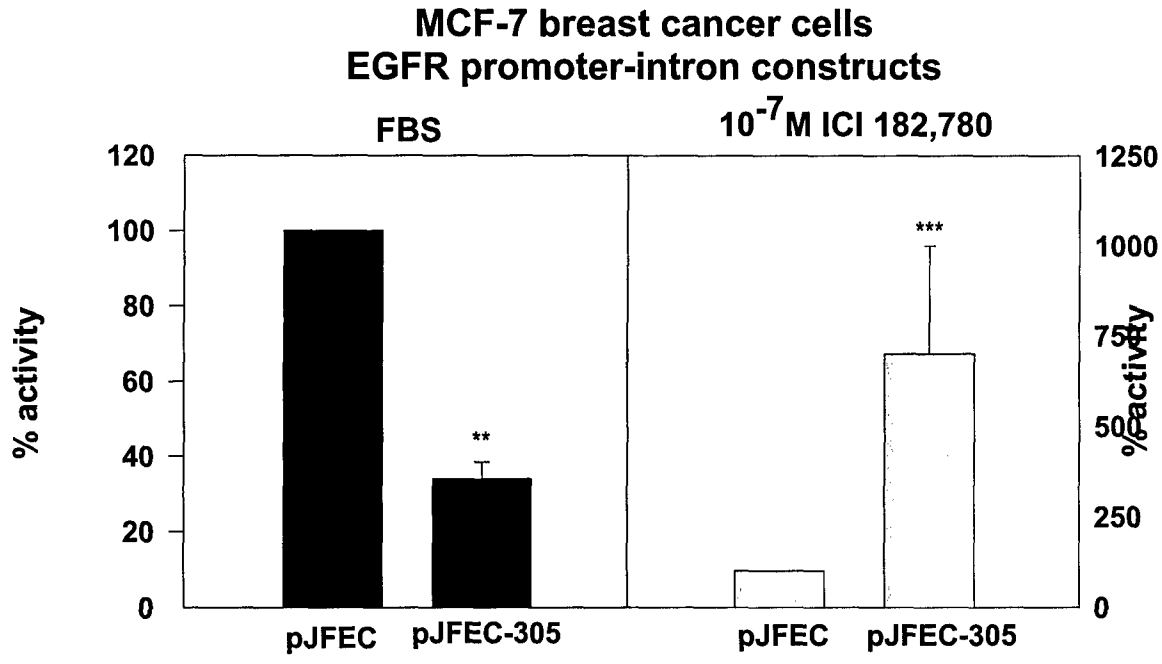

MCF-7 breast cancer cells
EGFR promoter-intron constructs
FBS
10-7M ICI 182,780
% activity
% activity
120
100
80
60
40
20
0
1250
1000
750
500
250
0
**
***
pJFEC
pJFEC-305
pJFEC
pJFEC-305

Figure 6. *The anti-estrogen, ICI 182,780, abrogates transcriptional repession mediated by the 305 bp intron negative regulatory element.*
Map of the EGFR gene first intron demonstrating the relative position of the 305 bp negative regulatory element. Transient transfections were performed with the EGFR-intron construct containing the 305 bp intron negative regulatory element in MCF-7 cells. Cells were untreated (FBS) or treated with 10^{-7}M ICI (10^{-7}M ICI 182,780) for 48 hours post-transfection. Activity is expressed relative to the parental construct (promoter alone) for each treatment condition. p values: **, p=0.01; and ***, p<0.001.

TRANSCRIPTIONAL REPRESSION OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) GENE EXPRESSION IN HORMONE-DEPENDENT BREAST CANCER CELLS

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The progression of breast cancer from an estrogen receptor positive (ER+), hormone-dependent to an estrogen receptor negative (ER-), hormone-independent phenotype involves the loss of ER expression and the up-regulation of a number of growth factor receptors and/or their ligands. The EGFR, one particular receptor overexpressed in breast cancer, demonstrates an inverse correlation with ER in a majority of breast tumors; however, a significant number of tumors co-express the two receptors. The expression of EGFR, independent of ER status, is indicative of a more aggressive phenotype and predicts for poor response to endocrine therapy and poor survival rate, suggesting that up-regulation of EGFR is involved in the progression of breast cancer to a more aggressive, hormone-independent phenotype.

Overexpression of EGFR in breast cancer cells occurs primarily due to transcriptional control. Both positive and negative regulatory factors play an important role in the regulation of EGFR gene expression in other cell lines, as well as in breast cancer cell lines. The main focus of our laboratory is the investigation of the differential regulation of EGFR gene expression in ER+ vs. ER- breast cancer cell lines. Experiments were designed to characterize the mechanism by which negative regulatory elements present within the EGFR gene first intron specifically repress EGFR gene expression in estrogen-dependent breast cancer cells, both functionally, utilizing transient transfection assays of reporter constructs, and through their ability to interact with protein factors, utilizing gel mobility shift and *in vitro* DNase I footprinting assays, as well as Southwestern analysis.

Transient transfections with reporter constructs have identified a 305bp intron element involved in the negative regulation of EGFR gene expression in hormone-dependent breast cancer cells. Furthermore, a 96bp fragment from this intron region demonstrated significant repressive activity in hormone-dependent breast cancer cells. In addition, this 96bp negative regulatory element demonstrated differential shift patterns in gel shift assays with nuclear extracts from ER+ vs. ER- breast cancer cell lines. Results from *in vitro* DNase I footprinting and Southwestern analysis suggest that multiple regulatory factors interact with the intron negative regulatory element. As a result, we hypothesize that *cis*-elements within the EGFR gene first intron are involved in the repression of EGFR gene expression in hormone-dependent breast cancer cells and that a complex of regulatory factors interacts with these intron elements and are involved in the transcriptional repression of EGFR gene expression.

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 1 Apr 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession document numbers be changed to "Approved for public release; distribution unlimited." Copies of these reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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